

## Real-Time Reverse Transcription-PCR for Detection of Rotavirus and Adenovirus as Causative Agents of Acute Viral Gastroenteritis in Children

Catriona Logan,<sup>1\*</sup> John J. O’Leary,<sup>2</sup> and Niamh O’Sullivan<sup>1,2</sup>

*Department of Microbiology, Our Lady’s Hospital for Sick Children,<sup>1</sup> and Department of Pathology, Coombe Women’s Hospital,<sup>2</sup> Dublin, Ireland*

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**Viral pathogens are the most common cause of gastroenteritis in developed countries. Human rotavirus and adenovirus infections are major causes of acute outbreaks and sporadic cases of gastroenteritis, occurring primarily among children less than 2 years of age. Patient hospitalization is often required, with enormous infection control implications. This work describes the development of real-time PCR assays for the detection of group F adenovirus, rotavirus A, and rotavirus C from stool specimens. Two hundred twenty stool samples from pediatric patients exhibiting symptoms of diarrhea and/or vomiting were examined. PCR results were compared with those of virus detection by electron microscopy and latex agglutination antigen detection. The incorporation of an internal-control RNA that was spiked into individual stool extracts functioned as an internal validation for the reporting of PCR-negative results. Rotavirus C was not detected by real-time PCR in the patient stool samples examined. Real-time reverse transcription-PCR resulted in 175% and 111% increases in the rates of detection of adenovirus F and rotavirus A, respectively, compared with latex agglutination testing. Molecular detection increased the number of stool specimens in which causative agents of gastroenteritis were identified by 155% compared to electron microscopy. Genotyping of a proportion of the rotavirus and adenovirus strains identified only genotype G1 rotavirus and both adenovirus genotypes 40 and 41 in circulation within the patient cohort examined. The results highlight the significance of rapid molecular methods for the routine screening of stool samples in hospital laboratories to provide rapid definitive diagnoses.**

Viral pathogens are the most common cause of gastroenteritis in developed countries (10, 11, 27). Worldwide, gastrointestinal rotavirus infections result in an estimated 440,000 deaths in children <5 years of age (18). Adenoviruses are second only to rotavirus as the most important causative agents of acute infantile gastroenteritis (31).

Rotaviruses are double-stranded RNA viruses, and serogroups A, B, and C are known to cause gastrointestinal infections in humans. Group A rotaviruses are predominant and result in severe diarrheal diseases in infants and young children (19). Group A rotaviruses can be classified on the basis of their viral protein 4-associated P type and viral protein 7 (VP7)-associated G type, of which G types G1, G2, G3, G4, G8, and G9 are most commonly detected (6). Group B rotaviruses cause adult diarrhea and are reportedly geographically confined, having been first identified in a large waterborne epidemic in China (5). Group C rotaviruses, an emerging cause of gastroenteritis in children over 2 years old and in adults, have now been identified as causative agents of gastroenteritis in both sporadic cases and outbreaks worldwide (8, 14, 23, 25, 28).

Human adenoviruses are double-stranded DNA viruses and are classified into six subgenera, A to F (30). Subgenus F (types 40 and 41) consists of the enteric adenoviruses and is an im-

portant cause of viral gastroenteritis among children less than 2 years of age (29), although older children and adults may also be infected.

Electron microscopy (EM) can be used to simultaneously detect and identify most enteric viral agents from a single stool specimen. However, the method is labor intensive and is relatively insensitive, requiring up to 10<sup>6</sup> intact viral particles per ml stool (22). Commercially available antigen detection kits, such as enzyme immunoassay (EIA) and latex agglutination (LA) tests, are often the method of choice for the diagnosis of adenovirus and rotavirus infection in stool samples in routine microbiology hospital laboratories. These kits are easy to use and highly specific; however, due to the levels of strain diversity among circulating gastrointestinal viruses, the estimated sensitivity for many antigen detection kits is low.

Various molecular techniques have been exploited for the development of highly sensitive and rapid assays for the detection of causative agents of viral gastroenteritis (16, 20, 26). Reverse transcription-PCR has reportedly increased the detection rate of rotavirus A by up to 48% compared to EIA or electron microscopy (2, 17). Simpson et al. report that the detection of adenovirus was increased by more than 200% using PCR compared to results with EM (27). The screening of stool specimens for a range of gastrointestinal viruses using highly sensitive molecular methods would provide an indication of the true prevalence of the causative agents of acute gastroenteritis in the pediatric patient cohort. In particular, rotavirus C prevalence remains largely unknown due to the

\* Corresponding author. Mailing address: Department of Microbiology, Our Lady’s Hospital for Sick Children, Crumlin, Dublin 12, Ireland. Phone: 00353-1-4085715. Fax: 00353-1-4085608. E-mail: catrionalogan@eircom.net.

TABLE 1. Nucleotide base sequences of primers and probes designed for development of real-time RT-PCR assays

Reaction (target gene)	Primer or probe (5' label)	Sequence (5'–3') <sup>a</sup>	Concn (μM) <sup>b</sup>	Position <sup>c</sup>
Adenovirus F (hexon)	Adeno.fwd	TTCCAGCATAATAACTCWGGCTTTG	0.60	2374–2398
	Adeno.rev	AATTTTTTCTGWGTCAGGCTTGG	0.60	2504–2482
	Adeno.probe1 (FAM)	CCATACCCCTTATTGG	0.10	2452–2468
	Adeno.probe2 (FAM)	CCTTACCCCTTATTGG	0.10	2452–2468
Rotavirus A (VP6)	RotaA.fwd1	GGATGTCCTGTACTCCTTGTCAAAA	0.60	26–50
	RotaA.fwd2	GGAGGTTCTGTACTCATTGTCAAAAA	0.60	26–51
	RotaA.rev1	TCCAGTTTGGAACTCATTTCCTCA	0.60	170–149
	RotaA.rev2	TCCAGTTTGGAAAGTCATTTCCATT	0.60	170–147
	RotaA.probe1 (VIC)	ATAATGTGCCTTCGACAAT	0.25	93–75
	RotaA.probe2 (VIC)	AATATAATGTACCTTCAACAAT	0.25	93–72
Rotavirus C (VP7)	RotaC.fwd	TTAGATACTACAAGTAATGGAATCGGATGT	0.60	655–684
	RotaC.rev	TGGGTGTCATTTGATACAACCTCA	0.60	731–708
	RotaC.probe (FAM)	CAGCTAGTACAGAACTT	0.20	689–706

<sup>a</sup> All probes are MGBNFQs with FAM or VIC as the reporter dye coupled on the 5' end of the oligonucleotide as indicated. Mixed bases in degenerate primers are as follows: W = A/T.

<sup>b</sup> Oligonucleotide concentration in real-time PCR amplification reactions.

<sup>c</sup> The position of each primer is relative to the numbering of the following gene sequences: adenovirus, relative to sequence with accession no. X51782; rotavirus A, relative to sequence with accession no. X94617; and rotavirus C position, relative to sequence with accession no. X77257.

absence of commercially available diagnostic tests. Routine diagnosis of rotavirus C is often inferred by an EM test positive for rotavirus but a negative group A rotavirus EIA result. A recent study showed that 43% of a population in the United Kingdom were seropositive for group C rotavirus, but a report investigating the occurrence of the virus in Irish patients has not been published (7).

This paper describes the design and development of a method for the routine detection of three gastrointestinal viruses from a single stool sample, namely group F adenovirus, rotavirus A, and rotavirus C. This research has developed a method for the efficient extraction of total nucleic acids from stool samples, with the incorporation of an internal positive control. In conjunction with the design of highly sensitive real-time reverse transcription (RT)-PCR assays, this has resulted in increased levels of detection of causative agents of gastroenteritis in the 220 pediatric stool samples examined compared to results obtained following electron microscopy and/or latex agglutination testing.

## MATERIALS AND METHODS

**Specimen collection.** A total of 220 stool samples were collected from patients exhibiting symptoms of diarrhea and/or vomiting received at the Microbiology laboratory, Our Lady's Hospital for Sick Children, from February 2004 to April 2005, divided into aliquots, and stored at  $-80^{\circ}\text{C}$  until analyzed using molecular methods. Twenty-five stool samples received at the laboratory from pediatric patients not exhibiting symptoms of gastroenteritis were similarly divided into aliquots and stored and used as controls for the purpose of this study.

**Latex agglutination detection of rotavirus and adenovirus antigens and electron microscopy.** All 220 stool samples were tested for the presence of rotavirus and adenovirus antigen using the Diarlex Rota-Adeno kit (Orion Diagnostica, Espoo, Finland). Specimens were processed as described in the kit protocol, using the centrifugation technique.

One hundred thirty-nine of the 220 stool specimens requesting additional viral investigations were sent to an external laboratory for examination by electron microscopy.

**Nucleic acid extraction from stool specimens.** Total nucleic acids were extracted using a single protocol similar to that published by O'Neill et al., which coextracts RNA and DNA for the isolation of viral nucleic acids from stool samples (16). All specimens were extracted in a dedicated class 2 laminar flow hood using dedicated pipettes and aerosol-resistant pipette tips. Fecal specimens

were prepared as 10% suspensions with STAR buffer (stool transport and recovery buffer; Roche Diagnostics GmbH, Mannheim, Germany). Chloroform aliquots of  $0.1 \times$  volume were added, and following mixing, the samples were centrifuged at  $8,000 \times g$  for 10 min. The aqueous layer (stool extract) was removed to a fresh tube and stored at  $-80^{\circ}\text{C}$ , or the extraction was completed before freezing. Stool extracts of 200 μl were further purified with the QIAGEN QIAamp DNA Blood mini kit as described for DNA extraction from whole blood in the handbook. Total nucleic acids were eluted from the spin columns using 50 μl of nuclease-free water. Extracted nucleic acids were stored at  $-80^{\circ}\text{C}$ . Stool samples were coextracted with a negative extraction control consisting of 200 μl of STAR buffer to which no stool had been added.

**Development of internal positive control system as a measure of the efficiency of removal of PCR inhibitors from stool samples.** To determine the efficiency of the extraction protocol in removing the high levels of PCR inhibitors present in stool samples, total mouse RNA was introduced to samples following stool clarification and carried through the specimen preparation, amplification, and detection protocols. Approximately 25 ng of mouse RNA was spiked into stool extracts. Following nucleic acid extraction and reverse transcription, each sample was assayed using the TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents (Applied Biosystems, Warrington, United Kingdom), a predeveloped real-time PCR assay for the detection of rodent GAPDH genes. PCR was carried out on the ABI Prism 7000 sequence detector (Applied Biosystems) using SDS software version 1.2. The  $\Delta Rn$  value of each sample (the normalized reporter signal minus the baseline signal), an indication of the magnitude of the signal generated in a given reaction, was determined. An extracted stool sample was considered inhibitory if the  $\Delta Rn$  value was at least 20% lower than the average  $\Delta Rn$  value for the extraction control.

**Reverse transcription reactions.** RT reactions were carried out in a final volume of 40 μl using the TaqMan reverse transcription kit (Applied Biosystems) on a GeneAmp 9700 thermocycler from Applied Biosystems. The reactions contained  $1 \times$  RT buffer, 5.5 mM  $\text{MgCl}_2$ , 0.5 mM each deoxynucleoside triphosphate (dNTP), 2.5 μM random hexamer, 0.4 U/μl RNase inhibitor, 1.25 U/μl Multiscribe reverse transcriptase, and 17.5 μl of extracted nucleic acids. Immediately before testing, extracted nucleic acids were removed from  $-80^{\circ}\text{C}$  storage and added to 200-μl thin-walled PCR tubes containing the RT buffer, dNTPs, and  $\text{MgCl}_2$ . To facilitate the reverse transcription of the double-stranded RNA of rotaviruses, the reaction components were denatured by heating at  $95^{\circ}\text{C}$  for 5 min and then snap-chilled on ice for 5 min. Random hexamer, RNase inhibitor, and Multiscribe reverse transcriptase were then added to the reaction. Thermal cycling parameters for the RT reactions were as follows: 10 min at  $25^{\circ}\text{C}$ , 30 min at  $42^{\circ}\text{C}$ , 20 min at  $48^{\circ}\text{C}$ , and 5 min at  $95^{\circ}\text{C}$ . Completed RT reactions were stored at  $-20^{\circ}\text{C}$  until required for further analysis.

**Primer and probe design for real-time PCR.** DNA sequences from highly conserved target genes were used for real-time PCR assay design as outlined in Table 1. Nucleic acid sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov) and aligned using the ClustalW (www.ebi.ac.uk/clustalw) multiple se-

TABLE 2. Oligonucleotide primers used for construction of positive-control plasmid DNA samples

Reaction and primers	Sequence (5'-3') <sup>a</sup>	Primer position <sup>b</sup>	Amplicon length (bp)
Adenovirus F			244
Adenovirus.ext.fwd	ATGTATTCCTTTTCCGAAACTTCCA	2284-2309	
Adenovirus.ext.rev	GCCACATGGTGCGATCGCA	2527-2509	
Rotavirus A			189
RotavirusA.ext.fwd	TTTAAAACGAAGTCTTCRACATGGAKGTCTGTA	4-37	
RotavirusA.ext.rev	TAATTGGTAGATTACCAATTCCTCCAGTTTG	192-162	
Rotavirus C			77
RotaC.fwd <sup>c</sup>	TTAGATACTACAAGTAATGGAATCGGATGT	655-684	
RotaC.rev <sup>c</sup>	TGGGTGTCATTTGATACAACCTCA	731-708	

<sup>a</sup> Mixed bases in degenerate primers are as follows: Y = C/T; R = A/G; K = G/T.

<sup>b</sup> The position of each primer is relative to the numbering of the following gene sequences: adenovirus, relative to sequence with accession no. X51782; rotavirus A, relative to sequence with accession no. X94617; rotavirus C position, relative to sequence with accession no. X77257.

<sup>c</sup> Primers used for the generation of a positive plasmid control for rotavirus C are as per real-time primers.

quence alignment package (3, 4). The following gene sequences were used to generate sequence alignments: adenovirus group F, accession no. X51782, D13781, and X51783; rotavirus group A, accession no. X94617, AF309652, AF079357, X94618, AB022768, U04741, and AF260931; rotavirus group C, accession no. X77257, X77258, M61100, and X77256.

All primers and probes were obtained from Applied Biosystems and were designed using Primer Express software version 2.0 (Table 1). Due to the amount of sequence heterogeneity, it was necessary to design degenerate primers for some targets, and in some cases a mix of primers and/or probes was required to ensure maximum sensitivity of the PCR assays (Table 1). During the course of this study, viral DNA and cDNA detection was carried out using 6-carboxyfluorescein (FAM)- or VIC-labeled linear MGBNFQ (minor groove binder/nonfluorescent quencher) probes.

**Real-time PCR amplification.** Each RT reaction sample was analyzed by PCR, in duplicate wells for all three viral targets. Multiple negative and positive controls were included for each PCR assay. Real-time PCR was performed on an ABI 7000 sequence detector (Applied Biosystems) using universal thermal cycling conditions: 2 min at 50°C (dUTP glycosylase step), 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Sequence detection software version 1.2 was used for all data analysis.

A typical 25- $\mu$ l real-time PCR amplification reaction contained 1 $\times$  TaqMan Universal Mastermix (containing AmpErase uracil-N-glycosylase, with dTTP partially replaced by dUTP), the appropriate forward and reverse primer(s) and MGBNFQ probe(s) at concentrations as detailed in Table 1, and 2  $\mu$ l of template RT reaction.

**Construction of plasmid DNA standards to determine PCR amplification efficiency and assay sensitivities.** The amplification efficiency and linearity range of the real-time PCR assays developed were demonstrated by amplifying duplicate aliquots of 10-fold serial dilutions ( $10^6$  to  $10^0$  copies) of the appropriate plasmid DNA standard. Plasmid DNA standards were prepared as follows: PCR products were generated using primers designed external to the real-time oligonucleotide primers (Table 2) or in the case of rotavirus C using the oligonucleotide primers designed for use in the real-time PCR assays. Solution phase PCRs contained (in a final volume of 50  $\mu$ l) 15 mM Tris-HCl, pH 8.0, 50 mM KCl, 15% glycerol, 200  $\mu$ M (each) dNTP, 2 to 4 mM MgCl<sub>2</sub>, 1.0  $\mu$ M of appropriate forward and reverse external primer(s), 1.25 U of Ampliqaq Gold DNA polymerase (Applied Biosystems), and 2 to 4  $\mu$ l of template DNA. For adenovirus F and rotavirus A, RT reaction samples identified as positive by real-time PCR were used as a template. An oligonucleotide longer designed for rotavirus C (a 93-base oligonucleotide corresponding to bases 647 to 739 of rotavirus C [accession no. X77257]) was synthesized by Sigma-Genosys (Suffolk, United Kingdom) and used as template DNA for the generation of plasmid DNA standards for rotavirus C.

Amplification reactions were performed on a GeneAmp PCR system 9700 (Applied Biosystems) with the following thermal conditions: initial denaturation at 95°C for 10 min; 45 amplification cycles with denaturation at 94°C for 30 s, annealing at 50 to 55°C for 30 s, and extension at 72°C for 90 s; and a final incubation at 72°C for 10 min. Amplified PCR products were analyzed by electrophoresis on 2% ethidium bromide-stained agarose gels and viewed under UV illumination. PCR products were cloned into pCR2.1-TOPO vector using the TOPO TA cloning system (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. The nucleotide base sequence of the insert DNA from each plasmid used as a DNA standard for real-time PCR was

determined by nucleotide sequencing of both DNA strands (Lark Technologies, Inc., Essex, United Kingdom).

**RNase and DNase digestion of nucleic acids extracted from stool specimens.** RNase and DNase digestion of a number of extracted stool samples was performed to verify the source of nucleic acid responsible for PCR amplification. Extracted stool samples that had been identified as positive for adenovirus F (DNA genome) or rotavirus A (double-stranded RNA genome) were examined. The RNase and DNase digestions were performed separately at 37°C for 30 min using RNase Cocktail and TURBO DNA-free (Ambion (Europe) Ltd., Cambridgeshire, United Kingdom), respectively, according to the manufacturer's instructions. Following reverse transcription, samples were examined by real-time PCR with the adenovirus F and rotavirus A real-time PCR assays.

**Differentiation of virus genotypes by PCR amplification and base sequencing.** A number of stool samples identified as positive for gastrointestinal virus(es) by real-time PCR were also examined with broadly reactive solution phase PCR assays (performed in a final volume of 50  $\mu$ l) for examination of the circulating virus strain diversity.

Adenovirus genotyping was performed using the method and primers (AD1 and AD2) of Sarantis et al. (24), a primer pair targeting conserved segments bracketing the hypervariable region 7 (HRV-7) of the hexon gene and suitable for the generic amplification of all adenovirus genotypes. Examination of the strain diversity of rotavirus A samples was performed using oligonucleotide primers described by DiStefano et al. targeting a region of known hypervariability in the VP7 gene (1). Amplified products were analyzed on 3% agarose ethidium bromide-stained gels. All RT-PCR products of the appropriate size were purified using the QIAGEN QIAquick PCR purification kit and cloned into the pCR2.1-TOPO vector using the TOPO TA cloning system (Invitrogen). The possibility of multiple genotypes of the same virus occurring in individual samples was not investigated. The nucleotide base sequence of the insert DNA from each plasmid was determined by nucleotide sequencing of both DNA strands, using the universal M13 forward and M13 reverse primers (Lark Technologies, Inc., Essex, United Kingdom). Sequence data generated from forward- and reverse-sequencing reactions was assembled, and a consensus sequence was imported into MEGA version 3.1 software for molecular typing determination by sequence homology (9). Phylogenetic analysis of sequence data was performed following multiple DNA sequence alignments of data obtained internal to the amplifying primers using ClustalW. A consensus phylogenetic tree was obtained using the neighbor-joining algorithm with a bootstrap value of 1,000 replications. Type assignment was based on the shortest distance from a prototype strain, as deduced from the phylogenetic tree.

**Nucleotide sequence accession numbers.** The nucleotide sequence data were submitted to GenBank. Partial VP7 gene sequences (rotavirus) were assigned accession numbers DQ498989 to DQ498997. Partial hexon gene sequences (adenovirus) were assigned accession numbers DQ498998 to DQ499005.

## RESULTS

**Development of an internal positive control protocol as a measure of the efficiency of removal of PCR inhibitors from stool samples.** All 220 stool samples examined by real-time PCR amplification using the TaqMan rodent GAPDH assay

were shown to have a  $\Delta Rn$  value of greater than 87% of that obtained for the corresponding extraction control. These data confirmed the efficient removal of PCR inhibitors from each individual sample.

**PCR specificity, sensitivity, and detection limits.** The specificity of each real-time primer and probe set used in this study was examined. Plasmid DNA standards ( $10^5$  copies of adenovirus F, rotavirus A, and rotavirus C) were examined with all three real-time PCR assays. Cross-reactivity of primer and probes was not observed when plasmid DNA standards were used as a template. Real-time PCR did not detect group F adenovirus, rotavirus A, or rotavirus C in the 25 control stool specimens examined.

To validate the real-time PCR assays prior to application to clinical samples, the detection limit and amplification efficiency of each reaction were determined. Standard curves with 10-fold serial dilutions of plasmid DNA controls (from  $10^6$  to  $10^0$  copies) were prepared and assayed in duplicate. The resulting standard curves (adenovirus group F, slope =  $-3.88$  and Y-intercept =  $43.74$ ; rotavirus group A, slope =  $-3.50$  and Y-intercept =  $44.34$ ; and rotavirus group C, slope =  $-3.56$  and Y-intercept =  $38.75$ ), with strong correlation coefficients ( $r^2 \geq 0.980$ ), indicated strong linear relationships over the 7-log range examined. PCR amplification efficiency for each assay was calculated from the slope of the standard curves (% efficiency =  $(10^{-1/\text{slope}} - 1) \times 100$ ) and ranged from 81.16% to 93.07%. A detection limit of 10 copies of target DNA per reaction was determined for all PCR assays, indicating high assay sensitivity.

**RNase and DNase digestion of nucleic acids extracted from stool specimens.** Total nucleic acids extracted from two stool samples were examined by real-time PCR prior to and following separate RNase and DNase treatment to confirm the source of nucleic acid responsible for PCR amplification. An adenovirus-positive sample (cycle threshold [ $C_T$ ] =  $17.37$ ) showed no change in  $C_T$  following RNase treatment ( $C_T$  =  $17.23$ ), while the sample was undetected by adenovirus real-time PCR following DNase treatment. Similarly, a rotavirus-positive sample ( $C_T$  =  $27.20$ ) was undetectable by real-time rotavirus PCR assays following RNase treatment, while DNase treatment did not significantly affect the  $C_T$  of the sample ( $C_T$  =  $27.79$ ).

**Detection of target viruses in stool specimens from patients with acute gastroenteritis.** The results of assaying all 220 pediatric stool specimens by real-time PCR (group F adenovirus, rotavirus A, and rotavirus C) and latex agglutination and of electron microscopy examination of 139 stool specimens are tabulated in Table 3.

The Diarlex Rota-Adeno kit identified 4 of the 220 stool samples examined as positive for adenovirus antigen (Table 3). Group F adenovirus was detected using real-time PCR in 11 of the 220 stool specimens, including the 4 specimens positive for adenovirus antigen. All 11 adenovirus-PCR positive specimens were from patients less than 2 years old.

Electron microscopy detected adenovirus particles in 4 of the 139 stool samples examined. Only one of these EM-positive samples was detected by real-time PCR for group F adenovirus ( $C_T$  =  $13.99$ ), a sample that was also positive for adenovirus by latex agglutination. However, real-time PCR detected an additional seven samples as adenovirus positive

TABLE 3. Detection of adenovirus and rotavirus in clinical samples by real-time RT-PCR<sup>a</sup>

Assay and parameter	Real-time PCR result					
	Adenovirus			Rotavirus		
	No. positive	No. negative	Total	No. positive	No. negative	Total
Latex agglutination						
No. positive	4	0	4	18	0	18
No. negative	7	209	216	20	182	202
Total	11	209	220	38	182	220
Electron microscopy						
No. positive	1	3	4	6	1	7
No. negative	7	128	135	14	118	132
Total	8	131	139	20	119	139

<sup>a</sup> Results of adenovirus group F and rotavirus group A detection by real-time PCR are compared to detection of rotavirus and adenovirus antigen by latex agglutination (220 stool specimens examined) and detection of rotavirus and adenovirus by electron microscopy examination (139 stool specimens examined).

that were not detected by electron microscopy (Table 3). Interestingly, two of these seven samples were also adenovirus positive by antigen detection.

Eighteen of 220 stool samples examined were identified as positive for rotavirus by latex agglutination testing (Table 3). Real-time PCR detected an additional 20 specimens as positive for rotavirus A. The patient age for rotavirus PCR-positive samples ranged from less than 1 month to 6.6 years old, with a mean patient age of 15 months. Over 80% (31 of 38) of rotavirus PCR-positive patients were less than 2 years old. Dual adenovirus-rotavirus infection was detected by PCR in 2 of the 220 specimens.

Seven of the 139 stool samples examined by electron microscopy were observed to contain rotavirus on examination. Six of these seven stool specimens were positive for rotavirus A using the real-time PCR assay designed as part of this study. In addition to the 6 EM-positive samples detected by rotavirus PCR assay, 14 specimens that were not detected by EM were identified as rotavirus A PCR positive (Table 3).

Rotavirus C was not detected by RT-PCR in the 220 stool specimens examined.

**Analyses of discrepant results.** Three of the 139 stool specimens examined by EM reported as containing adenovirus on examination by EM were repeatedly negative for group F adenovirus by real-time PCR (Table 3), including one specimen from a patient of >15 years of age. The possibility that adenovirus particles, perhaps serotypes other than type 40 or 41, were present was further investigated. Using the primers of Sarantis et al. that detect all known serotypes of human adenovirus with high sensitivity, solution-phase PCRs were performed. All three specimens failed to produce a PCR amplicon.

Six of the seven specimens positive for rotavirus by EM were detected with the rotavirus A real-time PCR assay designed in this study. The PCR negative, EM-positive stool specimen was reextracted and following reverse transcription reexamined by real-time PCR. No amplification signal was observed with the



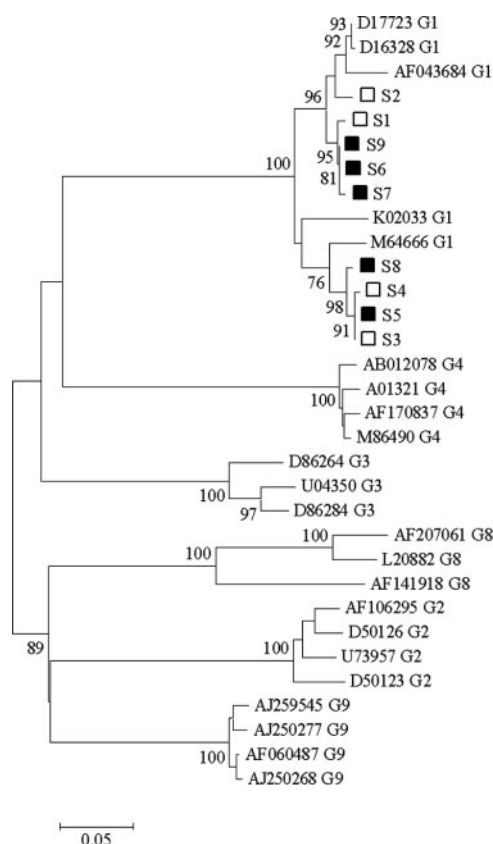


FIG. 1. Consensus phylogenetic tree derived from nucleotide sequence alignment of cloned amplicons generated by rotavirus genotyping PCRs and sequences available in GenBank. Tree was generated using MEGA 3.1 as described in Materials and Methods. Bootstrap values (from 1,000 replicates) are expressed as percentages for each node. DNA sequences obtained from GenBank are listed as the accession number and serotype number. DNA sequences S1 to S4, corresponding to clinical specimens identified as negative for rotavirus by latex agglutination testing, are indicated with a white square. Stool specimens corresponding to sequences S5 to S9 were positive for rotavirus by latex agglutination testing, as indicated by the shaded square. All clone sequences clearly cluster with G1 serotype rotavirus sequences.

rotavirus A or rotavirus C assays, while the internal control mouse RNA was detected within the defined limits. Attempts to amplify a PCR product, using the primers of DiStefano et al., with a solution-phase PCR assay for the amplification of all rotavirus serotypes also failed.

**Differentiation of virus genotypes by PCR amplification and base sequencing.** A total of eight stool samples identified as positive for adenovirus group F by real-time PCR were examined using the adenovirus serotyping method of Sarantis et al. The eight HVR-7 PCR amplicon sequences (internal to the primers) ranged in length from 538 to 541 bp and displayed 74 to 100% sequence identity with each other, with seven of the eight samples showing  $\geq 94\%$  sequence identity. Blast analysis of all eight amplicon sequences identified entries in the GenBank database with 99 to 100% sequence identity. Six of the eight clones had a top database hit with accession number AB103344 (type 41), while the top database hits for the remaining two clones were accession number AB103347 (type 41) and accession number

X51782 (type 40). A phylogenetic tree was generated from alignment of the nucleotide sequences of the amplicons obtained from clinical specimens and HRV-7 regions of hexon gene sequences currently in GenBank corresponding to adenovirus types 1 to 49 (24). Clustering of all eight clones obtained from clinical samples was observed with the group F adenoviruses. Seven of the eight clone sequences (GenBank accession numbers DQ498998 to 499004) were assigned as adenovirus type 41, and the remaining clone sequence was assigned as adenovirus type 40 (GenBank accession number DQ499005).

Nine stool samples identified as positive for group A rotavirus by real-time PCR were examined using the rotavirus VP7 typing method of DiStefano et al. (1). The cloned amplicons (internal to the primers) were 307 bp and displayed 91 to 100% sequence identity with each other. Blast analysis of all nine sequences identified entries in the GenBank database with  $\geq 98$  to 100% sequence identity. Phylogenetic analysis of VP7 gene sequences currently in GenBank corresponding to rotavirus G types 1, 2, 3, 4, 8, and 9 clearly identified each serotype as a distinct cluster, with strains of the same serotype clustering together (Fig. 1). All nine of clone sequences S1 to S9 (with corresponding GenBank accession numbers DQ498989 to 498997) from clinical specimens clearly clustered with G1 serotype rotavirus sequences.

## DISCUSSION

This work describes the development of molecular assays for the routine detection of three agents responsible for viral gastroenteritis from a single stool sample, namely group F adenovirus, group A rotavirus, and group C rotavirus. An assay for the detection of group B rotavirus was not developed due to the geographic confinement of the virus. A novel method was developed for the prevention of false negatives due to the high levels of PCR inhibitors present in stool samples with the incorporation of an internal control RNA that is spiked into individual stool extracts. The inclusion of a random hexamer-primed reverse transcription reaction in the protocol developed, while not necessary for the amplification of DNA templates, allows a single protocol for the analysis of stool samples for differing nucleic acids. Degenerate primers and in some cases a mix of primers and/or probes were used in PCRs to ensure maximum sensitivity of the assays. The alternative of lowering PCR temperature stringency conditions could have compromised assay specificity.

Rotavirus C was not detected by real-time PCR in the 220 stool samples examined, suggesting that the virus is not currently circulating as a commonly occurring causative agent of viral gastroenteritis in this Irish pediatric cohort.

Real-time PCR resulted in 175% and 111% increases in the rate of detection of adenovirus and rotavirus, respectively, compared to latex agglutination testing. Real-time RT-PCR detected all specimens identified as positive for rotavirus or adenovirus by latex agglutination or antigen detection.

Adenovirus serotypes 40 and 41 were identified, while all rotavirus specimens examined were assigned genotype G1. Genotyping studies of rotavirus samples recovered from infected children in Ireland have previously identified G types 1, 2, 3, 4, 8, and 9 (12, 13, 15, 21), with all studies identifying G1

as the predominant G type. The finding of only G1-type rotavirus strains in this study is therefore surprising, albeit only nine patient specimens were genotyped, all of which were received at the hospital over a period of less than 12 months.

Serotype specificity could not account for the failure of latex agglutination testing to detect adenovirus or rotavirus antigen in a number of PCR-positive stool specimens. Three of seven adenovirus specimens assigned to serotype 41 were negative for adenovirus by LA. Similarly, four of nine genotype G1 rotavirus specimens were negative for rotavirus by LA (Fig. 1). The viral protein-4-associated P type of rotavirus specimens was not examined.

Real-time PCR resulted in 100% and 186% increases in the rate of detection of adenovirus and rotavirus, respectively, compared to electron microscopy testing of the 139 stool specimens examined. These results compare favorably with those of previous studies, which report significantly increased levels of rotavirus and adenovirus detection by PCR compared to EM (17, 27).

Four stool specimens deemed positive for adenovirus or rotavirus by electron microscopy were not detected by real-time PCR or LA testing. All four samples also failed to produce an amplicon using the appropriate universal genotyping primers for adenovirus and rotavirus detection. The four specimens were subsequently screened using real-time RT-PCR assays for the detection of human astrovirus, norovirus, and sapovirus. One specimen was real-time PCR positive for norovirus genogroup 2 (C. Logan, J. J. O'Leary, and N. O'Sullivan, unpublished data).

Failure to amplify rotavirus and adenovirus EM-positive specimens both with the real-time PCR assays designed in this study and with PCR assays capable of detecting all serotypes of the viruses is unlikely, in particular given the relatively low sensitivity of EM. The possibility that these samples contained virus particles morphologically similar to rotavirus or adenovirus, but with little nucleotide sequence homology, cannot be ruled out. In particular, this highlights the problems associated with the determination of a specimen as rotavirus C positive based on a positive EM but negative rotavirus LA result.

In conclusion, this work reports the design, development, and application of real-time PCR assays for the rapid, specific, and highly sensitive detection of group F adenovirus and rotavirus groups A and C as causative agents in gastrointestinal infection. The results reported in this study highlight the inadequacies of nonmolecular methods currently used for the routine detection of gastrointestinal viruses and emphasize the importance of the application of rapid molecular methods to clinical services in hospital laboratories to provide definitive diagnoses. In conjunction with genotyping protocols, highly sensitive molecular methods could provide valuable information on the incidence of currently circulating viral strains, therefore lending itself to surveillance of viral transmission.

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